Zebrafish *pea3* and *erm* are general targets of FGF8 signaling

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Phenotypic analysis of both zebrafish and mouse has shown that fibroblast growth factor 8 (FGF8) is required for many developmental decisions. To further our understanding of the FGF8 signaling process, we sought to identify new transcriptional targets of the pathway. Here, we propose that two zebrafish ETS genes, *pea3* and *erm*, are general targets of FGF8 signaling, based upon the following observations: both genes are expressed around all early FGF8 signaling sources, both genes are downregulated in *fgf8* mutant embryos in all tissues known to require *fgf8* function, a pharmacological inhibitor of the FGF pathway completely abolishes expression of both genes, and ectopic expression of *fgf8* is sufficient to induce both genes. The finding that *pea3* and *erm* are common transcriptional targets of FGF8 signaling suggests that they are general mediators of FGF8 signaling during development. In addition, we observed that *pea3* is often expressed close to an FGF8 source, and *erm* is expressed in a broader domain. To test whether this differential expression is established by FGF8, we have induced FGF8 ectopically and show that it is sufficient to recapitulate the endogenous nested expression pattern of *pea3* and *erm*.

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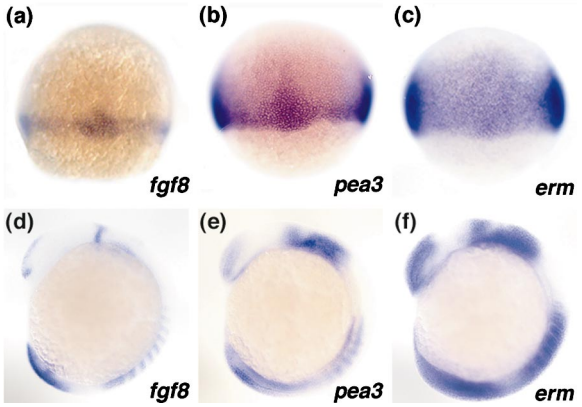
Results and discussion

PEA3 (polyomavirus enhancer activator 3) [1] and ERM (ETS related molecule) [2] belong to the PEA3 class of ETS-type transcription factors. Although the complex developmental expression patterns of *erm* and *pea3* in both mouse and zebrafish have been described [3–5], it is not clear how these expression domains are established. We observed a correlation between the expression of zebrafish *erm*, *pea3*, and *fgf8* and hypothesized that *erm* and *pea3* are general transcriptional targets of FGF8 signaling. Figure 1 compares the expression patterns of all three genes during two stages of development: at 60% epiboly, when *fgf8* is expressed at the leading edge of the gastrula in the epiblast (Figure 1a), and at the eight-somite stage, when *fgf8* is expressed in the forebrain, the mid/hindbrain boundary (MHB), the lateral plate mesoderm adjacent to the hindbrain, the anterior of each somite, and the tailbud (Figure 1d; [6]). Both target genes are expressed around these FGF8 signaling sources; however, their expression patterns are not identical. Whereas *pea3* is expressed close to these signaling sources, *erm* is expressed in a broader domain (Figure 1b,c,e,f).

To test whether *erm* and *pea3* are targets of FGF8, we first examined their expression in *acerebellar (ace)* mutant embryos, which carry a point mutation in the *fgf8* gene [6]. To summarize, both target genes are downregulated only in tissues known to be affected by the *acerebellar* mutation (Table 1). Furthermore, several tissues that were not previously identified as targets of FGF8 signaling also have diminished expression of both target genes in *acerebellar* embryos. Closer inspection of the derivatives of these tissues revealed that they are also affected by the *acerebellar* mutation (see below). The loss of expression of *erm* and *pea3* in tissues requiring FGF8 function suggests that these two target genes play a role in many different FGF8-mediated decisions.

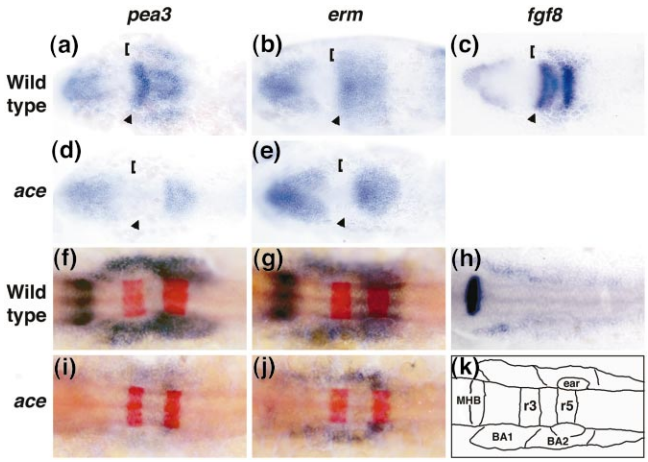
The complex expression patterns of both target genes in and around the hindbrain led us to examine this region more closely. Two of the earliest requirements for *fgf8* take place here: the formation of the MHB, and patterning of the heart field [6, 7]. At the three-somite stage, *fgf8* is expressed at the MHB, rhombomere 2 (r2), rhombomere 4 (r4), and in the lateral plate mesoderm bordering the heart field (Figure 2c). *pea3* and *erm* are expressed in tissues bordering these FGF8 sources, including the newly formed endoderm and neural crest, the neural tube, the lateral plate mesoderm, and the overlying ectoderm (Figure 2a,b; data not shown). In *acerebellar* embryos, expression of both target genes is reduced in tissues throughout this region, including the MHB and the heart field (Figure 2d,e). By the 17-somite stage, both target genes are strongly downregulated in *acerebellar* embryos. At this stage, the neural crest is migrating away from the neural tube, and the ear is forming as an ectodermal placode adjacent to rhombomere five. *fgf8* expression continues in the MHB and in the lateral plate mesoderm (Figure 2h). In wild-type embryos, both target genes are expressed in the neural crest, which will populate the first two pharyngeal arches, the ear placode, and the MHB; and, in addition, *erm* is expressed in the neural tube from r4 to

Figure 1



pea3 and *erm* are expressed in intimate association with FGF8 signaling centers. **(a–c)** A dorsal view of embryos at 60% epiboly. **(d–f)** A lateral view of embryos at the eight-somite stage. (b,e) *pea3* and (c,f) *erm* are expressed in and around tissues expressing *fgf8* (a,d). In the tailbud, in the somites, around the MHB, in the forebrain, and in the embryo during epiboly, *pea3* expression domains are nested within those of *erm*.

Figure 2



Downregulation of *pea3* and *erm* in *acerebellar* embryos. **(a–e)** In wild-type embryos, (a) *pea3* and (b) *erm* are expressed around FGF8 signaling sources (c) and are lost in the heart field and at the presumptive MHB in *acerebellar* embryos (d,e). Embryos are at the three-somite stage, brackets indicate the heart field, and arrowheads indicate the MHB. **(f–h)** At the 17-somite stage, *fgf8* is expressed at the MHB and in the lateral plate mesoderm (h). In wild-type embryos, (f) *pea3* and (g) *erm* are expressed around the MHB, in the ear placode, and in the neural crest, which will populate the first and second arch (BA1 and BA2, respectively). In addition, *erm* is expressed in the hindbrain from r4–r6 (g). In *acerebellar* embryos, expression of both target genes is strongly downregulated in all of these tissues **(i,j)**. **(k)** A camera lucida drawing of (g). *krox20*, shown in red, labels r3 and r5 in (g,h,j,k). All embryos in this figure are flatmount, dorsal views, with the anterior oriented toward the left.

r6 (Figure 2f,g). In *acerebellar* embryos, expression of both target genes is diminished in all of these tissues (Figure 2i,j).

The loss of expression of *erm* and *pea3* in the pharyngeal endoderm, the cranial neural crest, and r4–r6 suggests that the development of these tissues may also be required FGF8 signaling. To test this hypothesis, we checked if there are defects in the derivatives of these tissues in *acerebellar* embryos. Both the endodermally derived pharyngeal pouches and the neural crest–derived facial cartilages are reduced in size and are disorganized (Figure 3c–f); and, in the hindbrain, r4 is expanded and r5 is reduced in size (Figure 3a,b). The reduction of target gene expression presages all of these phenotypes, indicating that these tissues are early targets of FGF8 signaling.

Table 1

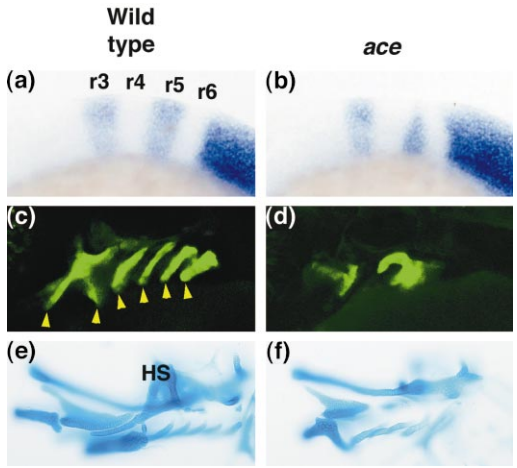
Reduction of *pea3* and *erm* expression in *acerebellar* embryos.

	Loss or reduction of target genes' expression in *ace* embryos	*ace* phenotype identified
Gastrulating embryo	no	no
Forebrain	yes	yes[26]
MHB	yes	yes[6]
Heart field	yes	yes[7]
Pharyngeal endoderm	yes	yes[d]
Cranial neural crest	yes	yes[d]
Otic placode	yes	yes[27]
r4–r6[a]	yes	yes[d]
Radial glia[b]	no	no
Pectoral fin bud	no	no
Somites	yes	yes[6]
Rohon-Beard neurons[c]	no	no
Tail bud	no	no

[a] r4–r6 express *erm* and not *pea3* [3].
[b] radial glia express *erm* and not *pea3* [3].
[c] Rohon-Beard neurons express *pea3* and not *erm* [3].
[d] phenotypes described in this paper.

The *acerebellar* phenotype is milder than that predicted by the complex expression of *fgf8*, suggesting that other FGF genes can substitute for *fgf8*. Consistent with this, two other FGF genes, *fgf4* and *fgf17*, are expressed in many of the same places as *fgf8* ([8, 9]; B. Draper, personal communication). Thus, redundancy between *fgf* genes may explain why many of the expression domains of *pea3* and *erm* are only diminished or unaffected in *acerebellar* embryos (Figures 2 and 4a,b,d,e). On the other hand, since many expression domains of both target genes are not near *fgf8*-expressing cells, *pea3* and *erm* transcription may not always depend upon FGF8 signaling (Figure 4a,d,g). To investigate these two possibilities, we treated early and late stage embryos (eight-somite stage and at 33 hr postfertilization) with an FGF receptor inhibitor, SU5402 [10]. After 6 hours in SU5402, expression of both target genes is completely turned off in domains bordering *fgf8*-expressing cells as well as in domains not neighboring

Figure 3



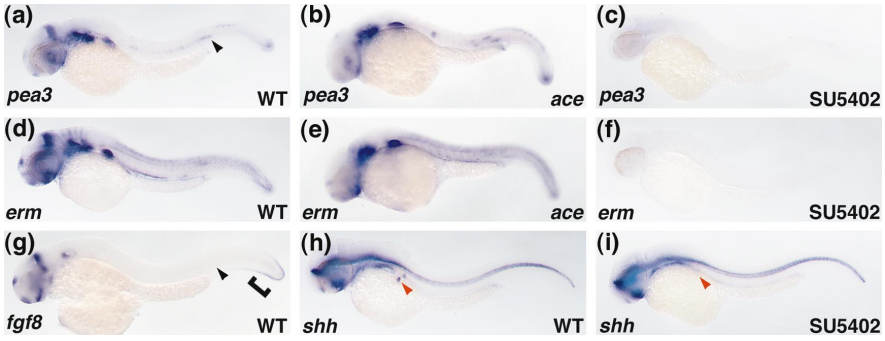
Additional phenotypes associated with the *acerebellar* mutation. **(a,b)** Whereas in wild-type embryos rhombomere segments are very equal in size (a), in *acerebellar* embryos, r5 is smaller, r4 is larger, and r3 is often also reduced in size (b). Embryos are at the 20-somite stage, *krox20* labels r3 and r5, and *hoxb4* labels the neural tube starting at r7. **(c,d)** Confocal images of living embryos at 36 hr postfertilization carrying a sonic hedgehog:GFP reporter construct [22]. The six endodermal pouches seen in wild-type embryos [yellow arrowheads in (c)] are reduced and disorganized in *acerebellar* embryos (d). **(e,f)** In *acerebellar* larvae, the neural crest–derived cartilages are variably reduced and disorganized (f), as revealed by alcian blue staining at day 5. In this mutant larva, the hyosymplectic cartilages (HS) are completely missing, and other cartilages are variably reduced. All the panels in this figure are lateral views, with the anterior oriented toward the left.

fgf8-expressing cells (Figure 4a,c,d,f; data not shown). This surprising result suggests that all expression of *erm* and *pea3* is FGF-dependent and that all tissues expressing either target gene are responding to an FGF signal.

Analysis of several vertebrate systems has suggested that FGF8 can act at a distance and can polarize tissues [11–14]. In agreement with this model, *pea3* is often expressed within 3–4 cell diameters from an FGF8 source, and *erm* is expressed up to 7–8 cell diameters away (Figure 1). To test whether FGF8 is sufficient to establish this nested pattern, we used a Drosophila heat shock promoter, hsp70, to ectopically express *fgf8* from a point source. Figure 5a,b shows that single cell clones expressing *fgf8* (black) are sufficient to induce *erm* or *pea3*, respectively (both red). Figure 5c,d shows two embryos that are hybridized with *fgf8* (dark red), *erm* (red), and *pea3* (black). In both embryos, *pea3* is expressed within 3–4 cell diameters from the FGF8 source, while *erm* is expressed up to 7–8 cell diameters away. These data suggest that transcription of both genes is differentially activated in response to a gradient of FGF8. However, an FGF gradient cannot be the only way in which the expression patterns of *pea3* and *erm* are established, since, for example, Rohon-Beard neurons express *pea3*, but not *erm* [3].

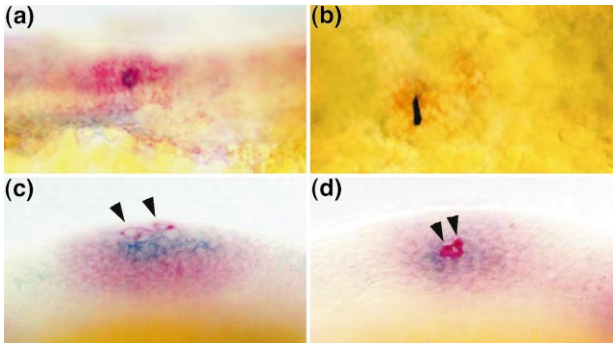
The finding that *pea3* and *erm* are general transcriptional targets of FGF8 signaling suggests that they are general mediators of FGF8 signaling during development. This is in contrast to the many tissue-specific targets of FGF8 signaling that have been identified. The only other known general transcriptional target of the FGF8 pathway is *sprouty* [15, 16]. Intriguingly, the *sprouty* gene product binds directly to components of RTK signaling pathways and is thus likely to be a component of the FGF signal transduction pathway [15, 17]. The third member of the PEA3 class of ETS proteins, *er81* (ETS-related 81) [18], has not been cloned from zebrafish. Its transcription in *Xenopus* animal caps is regulated by FGF, and its expression overlaps extensively with *pea3* and *erm* during mouse development [4, 19]. Thus, it is likely that *er81* is also a

Figure 4



A pharmacological inhibitor of the FGF pathway abolishes expression of *pea3* and *erm*. **(a,d,g)** Wild-type expression of *pea3*, *erm*, and *fgf8* at 36 hr postfertilization In *acerebellar* mutant embryos, many expression domains of **(b)** *pea3* and **(e)** *erm* are not reduced, suggesting redundancy between *fgf8* and other FGF genes. For example, the tailfin expresses *fgf8* along the periphery [bracket in (g)] but shows no reduction of *pea3* or *erm* in mutant embryos [compare (a) and (d) to (b) and (e)]. Furthermore, tissues expressing either target gene are not always neighboring FGF8 signaling centers, suggesting that other FGFs activate *pea3* and *erm*. For example, the lateral line primordia expresses *pea3* but is far from an FGF8 source [black arrowheads in (a) and (g)]. Embryos treated with SU5402 show no expression of **(c)** *pea3* or **(f)** *erm*, confirming that other FGF genes are responsible for the remaining expression of both genes in *acerebellar* mutant embryos. **(h,i)** As a control for the SU5402 experiment, we used *sonic hedgehog* (*shh*) which is only lost in the pectoral finbud (red arrowheads), where it is known to be a target of FGF signaling [23, 24]. Embryos were dechorionated at 29 hr postfertilization and incubated in 20 μM SU5402 (Calbiochem) for 6 hr at 29°C before fixation. Embryos treated with SU5402 at the eight-somite stage also showed a complete loss of target gene expression (data not shown).

Figure 5



Ectopic expression of *fgf8* induces nested expression of *pea3* and *erm*. Wild-type embryos were injected at the one-cell stage with a *hsp70:fgf8* fusion construct, allowed to develop for 12 hr, heat shocked at 37°C for 1 hr, then incubated an additional 3.5 hr at 29°C before fixation. When plasmid DNA is injected into zebrafish at the one-cell stage, the developing embryos show highly mosaic inheritance of the transgene. This allowed us to identify animals that had only a few cells carrying the *hsp70:fgf8* construct. After performing in situ hybridization, we searched for embryos containing small clones ectopically expressing *fgf8* in the posterior hindbrain, a region that does not express *fgf8* in wild-type embryos or either target gene at this stage. **(a,b)** A single cell expressing *fgf8* (black) is sufficient to induce (a, red) *pea3* or (b, red) *erm* in the surrounding tissue. Note that the *fgf8*-expressing clone does not induce *fgf8* in neighboring cells. **(c,d)** Expression of *fgf8* (dark red) from a small clone of cells (arrowheads) induces *pea3* (black) within 3–4 cell diameters and *erm* (red) within 7–8 cell diameters. The *hsp70:fgf8* clone in (c) is made up of two ectodermal cells, and the clone in (d) is made up of three cells within r6. Double in situ hybridizations were performed, essentially as in [25]. For (c,d), digoxigenin was used to label the *pea3* probe, and fluorescein was used to label both the *erm* and the *fgf8* probe. Cells expressing *fgf8* under the control of the heat shock promoter can be distinguished from the *erm*-expressing cells by their stronger staining intensity. All panels in this figure are views of the posterior hindbrain, with the anterior oriented toward the left.

common target of FGF8 signaling. The null allele phenotypes for both *er81* and *pea3* in mouse have recently been published, and neither phenotype is embryonically lethal [20, 21]. One explanation for this surprising result is that there is redundancy between the members of the PEA3 class. In light of our findings, we find it probable that double or triple mutant combinations between members of this class will yield stronger embryonic phenotypes.

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